Research Article

A Metabonomic Study of the Effect of Methanol Extract of Ginger on Raji Cells Using $^1$HNMR Spectroscopy

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Cancer is currently a major international health problem. The development of resistance to chemotherapy has resulted in the search for herbal drugs. Ginger is a medicinal plant with several clinical applications. Metabolomics is a simultaneous detection of all the metabolites by use of $^1$HNMR or mass spectroscopy and interpretation by modeling software. The purpose of this study was to detect the altered metabolites of Raji cells in the presence of ginger extract in vitro. Cells were cultured in the presence and absence of methanolic ginger extract in RPMI medium. IC$_{50}$ determined by MTT and lipophilic and hydrophilic extracts were prepared from control and treated groups which were analyzed by $^1$HNMR. The IC$_{50}$ was 1000 $\mu$g/mL. Modeling of spectra was carried out on the two groups using OSC-PLS with MATLAB software and the main metabolites detected. Further analysis was carried out using MetaboAnalyst database. The main metabolic pathways affected by the ginger extract were detected. Ginger extract was seen to affect the protein biosynthesis, amino acid, and carbohydrate metabolism and had a strong cytotoxic effect on Raji cells in vitro.

1. Introduction

According to GLOBOCAN (Cancer Incidence and Mortality Worldwide) 2012, an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008. Prevalence estimates for 2012 show that there were 32.6 million people (over the age of 15 years) alive who had had a cancer diagnosed in the previous five years. Projections based on the GLOBOCAN 2012 estimates predict a substantive increase to 19.3 million new cancer cases per year by 2025, due to growth and ageing of the global population. More than half of all cancers (56.8%) and cancer deaths (64.9%) in 2012 occurred in less developed regions of the world [1].

Cancer is treated with surgery, radiotherapy, chemotherapy, hormone therapy, biological therapy, and targeted therapy [2]. Current anticancer drugs used in chemotherapy are usually immunosuppressive and cause severe side effects [3], some of them resulting in drug resistance which is a major problem in cancer treatment process [4]. Studies have shown that plant sources of anticancer agents and their derivatives are very useful for the treatment or prevention of cancer in humans. These herbal compounds are important origins of clinically valuable anticancer agents. For example, they include alkaloids from the Madagascar periwinkle such as vinblastine which is used to treat Hodgkin’s lymphoma and vincristine for non-Hodgkin’s lymphoma and also the camptothecin derivatives from the bark and stem of Camptotheca acuminata, topotecan and irinotecan, used for lung and ovarian cancer [5].

Ginger has been mentioned in traditional medicine in China and India and has been used for more than twenty-five centuries [6]. It is recorded as a food supplement in the list of safe plants of FDA [7]. WHO has recognized ginger as a useful medicinal herb [8]. Several studies indicate the effect of this plant for treatment of different types of cancer cells including lung, ovarian, colon, breast, skin, prostate, and pancreatic cancers [9]. Ginger contains active
ingredients; the most important ones from the lipophilic extracts of its rhizome include gingerols, which can convert shogaol and zingerone to 4-paradol. The particular flavour of ginger depends on 6-gingerol and along with 6-shogaol is responsible for its medicinal activities including antipyretic, analgesic, antitussive, and antihypertensive effects as well as antioxidant, anticancer, anti-inflammatory, antiangiogenesis, and antiarteriosclerosis properties [10].

Metabonomics is a novel field dealing with simultaneous study of the entire metabolites in the body, resulting in the metabolic model of the cell using high throughput technology like $^1\text{HNMR}$ and LC-MS followed by advanced software and multivariate analysis methods. Metabonomics is widely used in toxicology and drug testing and has been used to study the difference between diseased and normal states in various body fluids, tissues, microbes, parasites, and cell lines. Its use in diagnosis of different ailments like arthrosclerosis, diabetes, and certain cancers is being studied [11].

The cell lines used in this study are Raji cells which are cultured from a line of lymphoblastoid cells derived from a human Burkitt (non-Hodgkin) lymphoma which is a cancer of the lymphatic system, particularly B lymphocytes found in the germinal center. These cells besides being used as a transfection host help understand the hematopoietic and other cell malignancies [12]. We have studied the effect of methanolic ginger extract on the metabolites of Raji cells in vitro using $^1\text{HNMR}$ spectroscopy.

2. Materials and Methods

2.1. Preparation of Ginger. Ground ginger powder was obtained from dried ginger from Mumbai, India. Its methanolic extract was purified using a Soxhlet extractor. In brief, 30 grams of ginger powder was loaded into the cartridge, 300 mL methanol was added, and extraction was started. The resulting extract was purified by the condenser and vacuum pump method. 0.7 grams of sticky extract was dissolved in 7 mL 0.01% DMSO (dimethyl sulfoxide) which gave 100 mg/mL. This was named as ginger stock.

2.2. Cell Culture. The Raji cell line (B-cell lymphoma) was supplied by the Pasteur Institute of Iran. It was cultured in a medium containing RPMI-1640 and 10% fetal calf serum, penicillin, and streptomycin. The cells were then incubated at 37°C with 5% CO$_2$ at 95% humidity.

2.3. Treatment of Raji Cells with Ginger. Three 12-well plates were selected. To each well, 1 mL of culture medium and 1.6 x $10^4$ Raji cells were added and treated with different concentrations of ginger 0.1%, 0.01%, and 0.001% of stock for 24, 48, and 72 hours. DMSO-solved ginger extract was diluted and added to 12-well plates in different concentrations. The test was carried out in duplicate. Control samples lacking the extract were cultured in each plate in duplicate.

2.4. Viability Test. Cells were counted for viability using trypan blue method at 24, 48, and 72 hours [13].

2.5. MTT Assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was also carried out at the same time points.

Briefly, 10,000 cells were placed in 96-well plates and ginger extract was added for 48 hours. The culture supernatant was then discarded, and the cells were incubated with 50 µg/mL MTT stock solution in PBS for 3 to 4 hours at 37°C. After adding 100 µL Formazan in methyl sulfoxide and shaking for 30 min the absorbance was read at 570 nm using Elisa-Reader instrument [14].

2.6. Cell Extraction. Methanol-chloroform-water extraction was performed as previously described [15]. The extraction procedure was performed on a crushed ice bath at 4°C. Briefly, cell pellets were resuspended in 500 µL of ice-cold 2:1 (v/v) methanol:chloroform solution and then transferred into a 1.5 mL Eppendorf tube. After vortexing, the tubes were incubated on a mixer for 10 min at 4°C. Then, 250 µL of ice-cold H$_2$O 1:1 (v/v) chloroform/H2O was added and mixed using a vortex mixer. The tubes were sonicated on ice for 10 min and centrifuged for 5 min at 18000 xg. The top hydrophilic and the bottom lipophilic extract were separated into different Eppendorf tubes. Water was removed from the sample by lyophilization, as presence of water will result in an additional peak in the $^1\text{HNMR}$ spectrum and cause interference in the spectra. The samples were lyophilized and stored at −20°C until analysis.

2.7. Preparation for $^1\text{HNMR}$. Lyophilized hydrophilic cell extracts were resuspended in 200 µL of buffer (150 mM potassium phosphate at pH 7.4, 1 mM NaN$_3$, and 0.01% and trimethylsilyl propionate (TSP) in 100% D$_2$O (deuterium oxide: the required quantity of buffer for each sample was originally prepared in H$_2$O, lyophilized, and reconstituted in 100% D$_2$O)), and the lipophilic cell extracts were resuspended in 200 µL deuterated chloroform. Both the extracts were analyzed by $^1\text{HNMR}$ analysis [15].

2.8. $^1\text{HNMR}$ Spectroscopy. The cell suspensions were placed in 5 mm probes (Bruker), for analysis. All $^1\text{HNMR}$ spectra were recorded on a Bruker spectrometer operating at 400 MHz spectroscopy by method 1D 1H CPMG (Carr-Purcell-Meiboom-Gill) spin-echo NMR. The temperature of the sample was maintained at 298 K. For each sample 128 transients and 16 dummy scans were collected into 32k data points of 45 min. The experiments were performed with a spectral width of 5200 HZ, acquisition time of 3.15 s, and relaxation delay of 1.5 s [15].

The $^1\text{HNMR}$ spectrum includes information about the effective metabolites present in the treated and control groups (both lipophilic and hydrophilic extracts of each). The spectra were then analyzed by MestReC Nova software. Correction of baseline was polynomial correction and automatic phase correction and chemical shifts were referenced to external 0.1% TSP in D$_2$O. All spectra were binned into 1000 parts and their normal intensity and chemical shift were entered into Excel file.
3. Data Processing

3.1. Chemometrics Analysis. The Excel files were entered into MATLAB 6.5 and PLS was implemented with the PLS-Toolbox version 3.0 analyzed by multivariate analysis methods using orthogonal signal correction and Partial linear square (OSC-PLS) [16].

3.2. Identification of Metabolites. Metabolites corresponding to these resonances were then identified using chemical shift assignments of spectra of metabolites of the cell extracts based on comparison with chemical shifts of metabolites in Human Metabolome Database Data Bank (HMDB) (http://www.hmdb.ca/metabolites) and in other published data. Analysis of metabolites was carried out using MetaboAnalyst software (http://www.metaboanalyst.ca/).

3.3. Statistical Analysis. Statistical analysis was carried out by SPSS version 19. The results are presented as mean ± SEM. Analysis of variations was done and comparisons between study groups were performed with ANOVA and Student’s t-test. Differences were considered significant at \( P < 0.05 \).

4. Results

The 48 h effect of alcoholic ginger extract on Raji cells and percent live cells are shown in Figure 1. IC\(_{50}\) of Raji cells is seen at 0.01% dilution of stock which is 1000 \( \mu \)g/mL.

The superimposed spectra of the hydrophilic phases of control and Raji cells exposed to methanolic ginger are seen in Figure 2. The greatest changes are seen in the 3.0-4.0 chemical shift. The superimposed spectra of lipophilic extract of control and Raji cells exposed to methanolic ginger extract and the greatest changes are seen in the 1.0-2.0 chemical shift areas (Figure 3). After this, the Excel files of normal intensity of the spectra were entered into MATLAB and OSC-PLS modelling was carried out in which only 1 orthogonal signal was removed.

Figures 4(a) and 4(b) show score plot OSC-PLS modeling for the two groups of control and drug treated in both hydrophilic and lipophilic extracts. Odd numbers indicate control group and even numbers are related to treated extract (treated with drug). A good separation is seen between the two groups in the two phases. The biplots of OSC-PLS with application for the two groups are shown in Figures 5(a) and 5(b).

The numbers of the altered metabolites from the figures correlated with the chemical shifts in the spectra in the above graphs are in fact the entries of altered metabolites. Using the reference database of HMDB the metabolites were identified. After processing the samples, the hydrophilic and lipophilic metabolites were obtained as shown in Table 1.

For identification of the affected pathways, the detected metabolites were inserted in the upload option of the MetaboAnalyst software. The affected metabolic pathways of the two phases are seen in Figures 6 and 7; the \( P \) values obtained using enrichment analysis are in descending order (Tables 2 and 3). The two different extracts obtained separated the metabolites into two groups: the lipophilic phase consisted mainly of...
amino acids and their metabolic cycles and the hydrophilic phase chiefly detected glucose cycles and their cycles. However, as both the phases are from the same cells, it can be concluded that ginger affects both amino acids and carbohydrate metabolism.

5. Discussion

The plants of the ginger family are widely included in diets throughout the world. The oleoresin extracted from the roots of ginger contains gingerol, which is a pharmacologically
Table 1: List of differentiating hydrophilic and lipophilic metabolites.

<table>
<thead>
<tr>
<th>Number in the biplot</th>
<th>Name of metabolite</th>
<th>Chemical shift and multiplicitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipophilic metabolites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>880</td>
<td>2-Ketobutyric acid</td>
<td>1.07 (t)</td>
</tr>
<tr>
<td>875</td>
<td>Isobutyryl-L-carnitine</td>
<td>1.14 (dd)</td>
</tr>
<tr>
<td>865</td>
<td>Isoleucine</td>
<td>1.24 (m)</td>
</tr>
<tr>
<td>895</td>
<td>Lactate</td>
<td>1.31 (d)</td>
</tr>
<tr>
<td>850</td>
<td>Homo-L-arginine</td>
<td>1.40 (m)</td>
</tr>
<tr>
<td>855</td>
<td>Lysine</td>
<td>1.54 (m)</td>
</tr>
<tr>
<td>Hydrophilic metabolites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>870</td>
<td>Isobutyryl-L-carnitine</td>
<td>1.14 (dd)</td>
</tr>
<tr>
<td>868</td>
<td>Isoleucine</td>
<td>1.24 (m)</td>
</tr>
<tr>
<td>528</td>
<td>Homo-L-arginine</td>
<td>1.63 (m)</td>
</tr>
<tr>
<td>653</td>
<td>D-Mannose</td>
<td>3.37 (dd)</td>
</tr>
<tr>
<td>624</td>
<td>Fructose-6-phosphate</td>
<td>3.64 (m)</td>
</tr>
<tr>
<td>526</td>
<td>Glucose</td>
<td>4.67 (d)</td>
</tr>
<tr>
<td>515</td>
<td>S-Adenosylhomocysteine</td>
<td>4.72 (t)</td>
</tr>
<tr>
<td>232</td>
<td>5-Methylcytidine</td>
<td>7.68 (s)</td>
</tr>
<tr>
<td>155</td>
<td>NADP</td>
<td>8.49 (s)</td>
</tr>
</tbody>
</table>

a: single, d: doublet, t: triplet, q: quartet, m: multiplet, and dd: double doublet.

Table 2: Overrepresentation analysis of hydrophilic phase.

<table>
<thead>
<tr>
<th>Metabolite sets enrichment overview</th>
<th>Total</th>
<th>Expected</th>
<th>Hits</th>
<th>Raw P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-alanine cycle</td>
<td>12</td>
<td>0.13</td>
<td>2</td>
<td>6.60E−01</td>
</tr>
<tr>
<td>Fructose and mannose degradation</td>
<td>18</td>
<td>0.20</td>
<td>2</td>
<td>1.48E−02</td>
</tr>
<tr>
<td>Glycolysis</td>
<td>21</td>
<td>0.23</td>
<td>2</td>
<td>2.00E−02</td>
</tr>
<tr>
<td>Galactose metabolism</td>
<td>25</td>
<td>0.27</td>
<td>2</td>
<td>2.79E−02</td>
</tr>
<tr>
<td>Gluconeogenesis</td>
<td>27</td>
<td>0.29</td>
<td>2</td>
<td>3.22E−02</td>
</tr>
<tr>
<td>Glutathione metabolism</td>
<td>10</td>
<td>0.11</td>
<td>1</td>
<td>1.04E−01</td>
</tr>
<tr>
<td>Betaine metabolism</td>
<td>10</td>
<td>0.11</td>
<td>1</td>
<td>1.04E−01</td>
</tr>
<tr>
<td>Nicotinate and nicotinamide metabolism</td>
<td>13</td>
<td>0.14</td>
<td>1</td>
<td>1.34E−01</td>
</tr>
<tr>
<td>Amino sugar metabolism</td>
<td>15</td>
<td>0.16</td>
<td>1</td>
<td>1.53E−01</td>
</tr>
<tr>
<td>Pentose phosphate pathway</td>
<td>18</td>
<td>0.20</td>
<td>1</td>
<td>1.81E−01</td>
</tr>
<tr>
<td>Protein biosynthesis</td>
<td>19</td>
<td>0.21</td>
<td>1</td>
<td>1.90E−01</td>
</tr>
<tr>
<td>Insulin signalling</td>
<td>19</td>
<td>0.21</td>
<td>1</td>
<td>1.90E−01</td>
</tr>
<tr>
<td>Methionine metabolism</td>
<td>24</td>
<td>0.26</td>
<td>1</td>
<td>2.34E−01</td>
</tr>
<tr>
<td>Valine, leucine, and isoleucine degradation</td>
<td>36</td>
<td>0.39</td>
<td>1</td>
<td>3.32E−01</td>
</tr>
</tbody>
</table>

Figure 6: Enrichment analysis indicating important metabolic pathways in the hydrophilic phase in descending order.

active substance [17, 18]. The impact of these substances on proliferation inhibition of human cancer cells through apoptosis pathway has been demonstrated [19]. Many herbs and spices have pharmacological and biochemical properties, including antioxidant and anti-inflammatory effects, which appear to be involved in anticancer and antimutagenic activities in a cell [20]. More than 50 antioxidants have been isolated from the ginger rhizome [21]. The most important antioxidant of ginger is [6]-gingerol, which has a sharp taste with significant antioxidant properties. Stimulation of the inhibitory phospholipid peroxidation in FeCl ascorbate system has been demonstrated [22]. 6-Gingerol has
an inhibitory effect on the xanthine oxidase system [23] which is responsible for production of reactive oxygen species such as superoxide anion. In another study, the inhibitory effect of gingerol on arachidonic acids causing platelet aggregation and formation of thromboxane B2 and prostaglandin D2 has been demonstrated [24]. Gingerol, shogaol, and similar components in ginger inhibit the biosynthesis of leukotrienes and prostaglandins by inhibiting 5-lipoxygenase and prostaglandin synthetase pathways [25].

Studies have shown that 6-gingerol and 6-shogaol are responsible for therapeutic activities including antiinflammatory, analgesic, antitussive, and antihypertensive effects. In addition, they have antioxidant, anticancer, anti-inflammatory, antiangiogenesis, and antiatherosclerotic properties [26, 27]. Notably, these compounds cause a low expression level of the gene regulating NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) which is a protein complex that controls transcription of DNA and is involved in cell proliferation and angiogenesis, resulting in apoptosis induction. In addition, they lower the level of IL-8 (interleukin-8) and VEGF 21 (vascular endothelial growth factor 21). Ginger extract inhibits platelet aggregation and thromboxane synthesis in vitro. This causes concerns about prolonged bleeding, but several European studies indicate that oral consumption of ginger has no significant anticoagulant effect [28].

Earlier studies carried out by us have shown that methanolic ginger extracts affect the sialic acid bond to О linked glycoproteins and mannose binding glycoproteins in Raji cells [29]. This study has shown the inhibitory effect of methanolic ginger extract on Raji cells, as proved by MTT and cell counting assay. The main metabolic cycles with the best P values which have changed in the Raji cells due to methanolic ginger extract are as follows: protein biosynthesis, glucose-alanine cycle, fructose and mannose degradation, glycolysis, and biotin metabolism.

5.1. Protein Biosynthesis. Involving the metabolites of L-lysine and L-isoleucine, these metabolites have been previously reported to play an important role in different cancers. Studies have shown that L-leucine or L-isoleucine supplementation enhanced growth of bladder urothelial tumors in rats by triggering expression of amino acid transporters and tumorigenesis-associated genes [30]. Methylation and acetylation of L-lysine seem to be important in promotion of proliferation of tumor cells. A report states that enhanced lysine methylation of HSP70 promoted proliferation of cancer cells through activation of a kinase enzyme named aurora kinase B [31]. Another study has shown that lysine acetylation promotes tumor growth through activation of 6-phosphogluconate dehydrogenase [32].

Lysine is also seen to participate in biotin metabolism [33]. Biotin uptake is described in small cell lung cancer cells, and it is seen that expression of oncogenes depends on it. Biotin uptake by breast cancer cells is higher as compared to normal cells for maintaining their high proliferative status [34]. This status is maintained by activation of oncogenes and loss of tumor suppressors which change metabolism and induce aerobic glycolysis. NADPH detected in our study acts along with glutamine, glucose, and ATP via the glycolysis pathway to provide the carbon skeletons to build new cancer cells [35].

As mentioned earlier, the effect of gingerol and shogaol on lowering the expression of the gene regulating protein biosynthesis such as NF-κB has been described. In recent years, the focus of cancer research is slowly shifting to the study of alteration of protein production, cell proliferation, cell volume, and/or biomarkers of protein synthesis which may help predict response to drugs targeting cancer metabolism [36].

Fructose and mannose degradation in which fructose-6-phosphate and D-glucose were detected was seen in our enrichment analysis. Fructose is reported to be significant in breast cancers and renal cell carcinomas [37, 38]. Ginger has an effect on these pathways in Raji cells.

The next important carbohydrate pathways are the glucose-alanine pathway and glycolysis which were marked by D-glucose and NADP and D-glucose and fructose-6-phosphate, respectively. As mentioned above, bioenergetics of cancer cells have shown that glucose is the main energy source for body cells and tumor cells consume a high level of glucose [39]. Changing glucose metabolism is one of the hallmarks of cancer. In 1929, Otto Warburg was the first to demonstrate that, unlike somatic cells, cancer cells use glucose in the anaerobic pathway, and there is an increase in lactate production rather than oxidative phosphorylation and ATP production. As a result, the pH of the surrounding tissues is decreased, further damaging DNA repair mechanisms [40]. Therefore, lactate which is detected in Table 1 is reported as a key intermediate in tumor metabolism, and most tumor cells use lactate for energy consumption. Tumor cells have a high glycolytic activity due to increased glycolysis [41].

![Figure 7: Enrichment analysis indicating important metabolic pathways in lipophilic phase P values in descending order.](image-url)
the later stages, the lactate produced is delivered to the liver and muscles and is converted to glycogen and intermediate metabolites of Krebs cycle are reduced [42]. The level of glucose is reduced because of its high consumption. There is increased turnover and activity of membrane phospholipids. According to other studies, there is an increase in the level of pyruvate and lactate in sera of cancer patients due to high consumption of energy and glycolysis. Ginger affects the glycolytic cycle as shown in our studies, and reports have shown its effect on activity of glycolytic enzymes in diabetic patients [43].

6. Conclusion
Methanolic ginger extract has an inhibitory effect on Raji cells even at low concentrations of 1000 μg/mL. Of the metabolites detected, lactate is reported as the key in tumor metabolism. Of the different cycles, proteins and carbohydrates metabolism were affected. In fact, the cycles detected by our metabonomic study have been reported earlier in genetics and immunology studies of ginger.

Conflict of Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

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